Clinical diagnostic significance of tumor suppressor gene methylation in oral mucosal lesions

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Abstract

Multiple genetic and epigenetic changes in critical regulatory genes have a role in the development of oral cancer. When genes are silenced by abnormally high levels of promoter hypermethylation, they have the potential to serve as biomarkers for early diagnosis, tumor molecular subtyping, prognosis and treatment monitoring. Different liquid biopsies have been found to have abnormal DNA methylation, which may be a viable alternative to solid biopsies. Noninvasive mouth cancer screening and early diagnosis could benefit from saliva testing for methylation genes. Gene promoter hypermethylation in saliva is examined here.

Introduction

Cancer is the third largest cause of death worldwide. Among that oral cancer is a serious health issue. Oral Squamous cell carcinoma (OSCC) is one of the most extensively frequent cancer types in developing countries, particularly India, and is connected with cigarette and alcohol consumption (Asokan et al., 2014). More than 300,000 people die each year from head and neck squamous cell carcinoma (HNSCC), which is diagnosed every year with 780,000 new cases. Hepatocellular carcinoma of the salivary glands (HNSCC) is frequently linked to a poor prognosis. Most squamous cell carcinomas are found in the oral cavity, where they account for 90 percent of all cancers with a dismal 5-year survival rate. Because of the lateness of the diagnosis, this patient's prognosis is bleak. Human papillomavirus (HPV) infections have been linked to 30 to 50 percent of cases of head and neck cancer. Biomarkers that are both sensitive and specific are needed to detect oral HNSCCs in high-risk patients because there are currently no unambiguous early warning indications. To detect HNSCC, an expert clinical examination of the upper airway and histological investigation of suspicious areas is now used; however, lesions can stay undiscovered in concealed places such as the base of the tongue and tonsils, which are difficult to access. In order to reduce the mortality and morbidity associated with HNSCC, it is critical to create diagnostic techniques that can detect the disease early on and allow for effective and timely therapies. More than a million people die each year from cancer-related causes due to tobacco use, which accounts for 30 percent of all cancer-related fatalities worldwide. One out of every three persons on the planet, according to a World Health Organization assessment, is nicotine dependent. Approximately 45 percent of male HNSCC cases and 75 percent of female HNSCC cases are attributed to smoking (Ovchinnikov et al., 2012).

The most common kind of head and neck SCC (HNSCC) is oral squamous cell carcinoma (OSCC), which is aggressive and frequently leads to local invasion and early lymph node

metastases. Despite significant improvements in OSCC treatment, the overall 5-year survival rate for OSCC is around 60% and has seen very modest improvement over the last two decades. In terms of epigenetic regulation and clinical applicability, there has been a paucity of understanding of OSCC carcinogenesis, development, and metastasis throughout the previous decade. As a result, identifying molecular changes in oncogenes or TSGs linked to OSCC will aid in better diagnosis predictions and early treatment (Marur and Forastiere, 2016).

Gene methylation

Cancer originates by way of two significant modifications namely genetic alterations and epigenetic abnormalities. The genetic abnormalities together with epigenetic processes like promoter hypermethylation influence the tumour suppressor genes causing loss of function. Several kinds of cancer have been linked to hypermethylation in tumour suppressor genes (Asokan et al., 2014). Tobacco smoking has been linked to alterations in the genome's methylation status, resulting in hypomethylation and the possible destabilisation of numerous repetitive, retro-transposon-derived, sequences. A well-known epigenetic event in cancer, hypermethylation of CpG islands in tumour suppressor genes promotes repression of their expression, and smoking can also induce hypermethylation of these CpG islands. The cell cycle regulator p16INK4a, DAPK1, and RASSF1A, all of which are known to modulate apoptosis, are often methylated in HNSCCs (Ovchinnikov et al., 2012).

Differentiated phenotypes result from epigenetic alterations such as DNA methylation and a wide variety of histone modifications. As the most researched epigenetic change, DNA methylation has a critical function in the development of mammals, but it is also linked to various disorders, including many types of human cancer. In cancer, gene promoter hypermethylation can quiet gene expression and influence biological processes, particularly tumour suppressor genes (TSGs), which play a crucial role in cancer start, development, and metastasis. Malignancies are known to have aberrant DNA methylation, and the methylation profiles of certain tumours may be linked to clinical outcomes. In colorectal cancer, promoter hypermethylation regulates transcriptional suppression of tumour suppressor candidate genes. Using high-throughput genome-wide methylation experiments, researchers can learn more about DNA methylation and how it affects gene expression (Kim et al., 2019).

Methylation of Tumor suppressor gene (TSG)

Oncogenic mutations of normal genes or the inactivation of tumour suppressor genes are to blame for a large number of malignancies. This can be done by the DNA methylation process,

which involves an enzyme called DNA Methylase adding methyl groups to locations near or on the suppressor gene itself. process of gene methylation called 'Epigenetic' refers to the fact that there is no change in the DNA's sequence (Baylin and Jones, 2011). In addition to lung, breast, and pancreatic cancer, some well-known tumour suppressor candidate genes have been used in numerous investigations. These TSGs, however, have not been studied in relation to their DNA methylation patterns in oral squamous carcinoma cancer (OSCC). This suggests that the epigenetic profile of CRC and other cancers may be shared by oral bacteria, based on previous results in this area (Kim et al., 2019).

Both the stochastic model and the informative model explain how tumour suppressor genes can be methylated. Tumor suppressor genes are methylated in the stochastic model, which is implicitly preferred in the literature. The resulting cells are more likely to grow. Hemimethylation occurs during the replication process of DNA, in which the DNA is split into two separate molecules, with one methylated and another unmethylated. This pattern is then passed on to the following generation by DNA methylases, which continue to modify the unmethylated segments of DNA. When considering the stochastic model, it's important to keep in mind that the methylation sites on tumour suppressor genes are frequently near together, and it's not clear how this might happen through a chance process. Using the instructional model, an oncogene initiates a series of precise biochemical steps that culminate in the DNA methylation of the tumour suppressor gene. Tumor suppressor genes are either particularly targeted by the DNA methylase, or non-specific locations across the genome are non-specifically methylated. In the same way as histone acetylases and other chromatin-modifying activities are involved in transcriptional regulation processes, such targeted or non-targeted modes of action are similar. Furthermore, typical transcriptional activation and deactivation methods require the recruitment of chromatin-modifying activities to specific genes, wherein they form local domains of histone modifications and either promote or reduce transcription (Struhl, 2014).

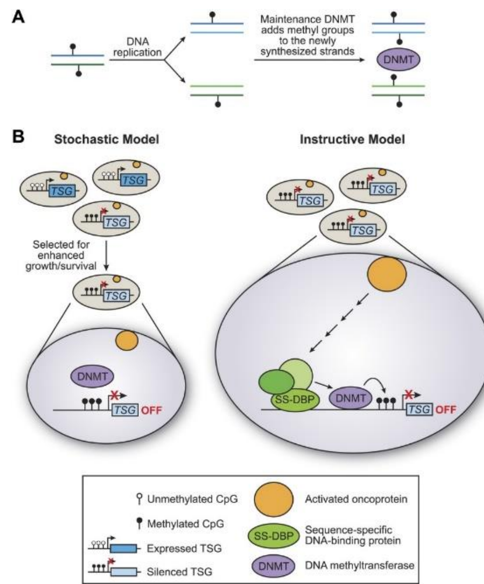

Figure 1: DNA methylation models for tumour suppressor genes that are both stochastic and informative. DNA methylation models for tumour suppressor genes that are both stochastic and informative. (A) DNA is methylated at CpG sites prior to replication on both strands. A new strand of DNA is created, but the original strand is not methylated. In order to preserve the methylation pattern at this site for future generations, DNA methylases (DNMT) add additional methyl groups to the hemi-methylated CpG sites. For example: (B) In the stochastic model (left), the cell that has both TSG silenced by chance and methylation of CpG sites (CpG sites) is selected for increased growth and survival. methylases maintain the methylation of CpG residues as detailed in (A). DNA methylation of the tumour suppressor gene is regulated in the teachable model by an activated oncoprotein (yellow circle), which activates a co-repressor complex (green circles) and a specific DNA binding protein (SS-DBP). DNA methylation is lost and the tumour suppressor gene is overexpressed when any component of this pathway is disrupted. According to the findings of Serra et al., the pathway begins with an activated KRAS enzyme (shown above as a yellow circle) boosting the production of PRKD1 and its substrate USP28 deubiquitinase. Phosphorylated USP28 deubiquitinates a transcription factor termed ZNF304, therefore boosting its concentration in the nucleus and thereby promoting cell proliferation. When this transcription factor (SS-DBP) attaches to a specific region of DNA, it brings together KAP1, an enzyme that methylates histones, and DNMT, an enzyme that methylates DNA (Kim et al., 2019).

Gene methylation and cancer development

For the first time, in 1994, Herman and colleagues demonstrated that hypermethylation of the promotor of the tumour suppressor gene VHL can silence it, resulting in renal carcinomas. Promotor hypermethylation is a frequent strategy for suppressing tumour suppressor genes in human malignancies, and it is considered to be as common as mutation (Shi et al., 2015). Even among people who are not showing any symptoms of cancer at this time, DNA methylation may be a major link between environmental factors and cancer, as there appears to be a correlation with lifestyle and cancer-related DNA methylation. DNA methylation is therefore expected to be a major factor in the genesis of tumours in a significant number of cancer patients. Because localised hypermethylation in promoters and other regulatory sites, allegedly produced by elevated quantities of the enzyme DNA methyltransferase, is the best technique to understand how DNA methylation influences carcinogenesis, tumour suppressors are silenced using this method. However, cancer can also be caused by abnormal methylation in other ways. Numerous malignancies have exhibited decreased methylation at oncogene promoters. Chromosome instability, mediated by genome wide hypomethylation, is another common mechanism (Hentze et al., 2019). It is the most common and well-known epigenetic modification of the human genome, with DNA methylation of gene promoters. The methylation of DNA sequences in CpG islands [repetitive dinucleotide sequences (5'-CG-3')] is not a byproduct of normal gene expression. CpGs make up around half of genomic sequences, and about 70–80 percent of them are methylated. Gene expression is regulated post-replication by DNA methyltransferase (DNMT) activity, which maintains the stable methylation pattern in the cell genome. There are many genes that encode tissue-specific proteins, however the CpG islands associated with these genes are usually methylated. Progeny cells inherit the DNA methylation pattern, which is genetic information about gene expression following cell division. Cell type-specific DNA methylation patterns appear to be an important predictor of gene expression patterns across tissues. As a result, the 5' regions of tumour suppressor gene promoters, which include a high concentration of CpGs sequences, are not methylated. Gene promoter regions must be unmethylated in order for gene transcription to be active and controlled (Jin et al., 2011). Gene silence is caused by methylation of the promoter region, which affects the structure of chromatin and turns it into a compressed and inactive state (heterochromatin). It prevents transcription factors from recognising the promoter transcription start site (TSS) and so the transcription of genetic information from DNA to mRNA is prevented. The genetic repression of information contained in DNA may be caused

by alterations in methylation patterns found in tumour cells. It's important to note that tumour cells' CpG methylation patterns are not the same as those found in healthy cells, and this can lead to the formation of distinct new DNA-methylation patterns. An epigenetic change in tumour cells has been found to be hypermethylation of tumour suppressor gene promoters. Normal cells do not methylate gene CpGs promoter regions because of their critical role in cell cycle control. There are other defensive mechanisms in non-tumor cells that regulate replication, chromatin remodelling, and DNA demethylation to counteract promoter hypermethylation. DNMTs are effectively prevented from gaining access to DNA by the processes referred to in this article. On the other hand, tumour cells lack mechanisms that prevent methylation, allowing DNMTs to evade these mechanisms. Overexpression of de novo promoter methylation-inducing genes (DNMT) in tumour cells is also a typical finding (Powrozek and Malecka-Massalska, 2016).

Gene methylation and cancer diagnosis

There are numerous advantages to DNA methylation over other molecular markers. Cancer-related DNA methylations in patients' serum were first detected in 1999, and it was later shown that DNA methylations are chemically and physiologically durable, even on cell-free cancer DNA in the blood. DNA methylations are a chemical and biological marker for cancer. 31-35

Genes that have methylation patterns are easier to identify than mutations because the binary signals may be amplified by methylation specific PCR-based approaches, which are more stable than those that have mutations. Because DNA methylation studies can concentrate on CpG sites, scanning the entire gene for alterations is not necessary. By changing its activity in a detectable way that may be linked to disease progression, a molecular marker might provide useful information in a diagnostic or prognostic scenario." Since DNA methylation changes have been linked to early carcinogenesis, even before tumour formation, as well as metastasis and treatment sensitivity, DNA methylation patterns may be used as both prognostic and diagnostic indicators (Hentze et al., 2019).

In the last four years, researchers have gained a great deal of insight into how methylation patterns are altered in human malignancies. Different genes have been found to exhibit methylation alterations that are particular to tumours. Cancer diagnosis, prognosis, and treatment could benefit from this information. Sulfite conversion, cDna microarray, and restriction landmark genomic scanning are just some of the newer techniques for detecting methylation that have emerged recently. Use the sodium bisulfite technique to identify normal

and abnormal methylation profiles. Unmethylated cytosines are converted to uracil by bisulfite, but methylated cytosines are unaffected. CpG island methylation can be studied using a variety of techniques after bisulfite modification. Methylation-sensitive single nucleotide primer extension and single-strand conformational polymorphism are a few of the techniques that can be used to better understand the genome's methylation patterns. Primers for DNA that has been bisulfite-treated can be designed using software. The actual methylation detection method must be sensitive, quick, straightforward, and reproducible in order to be helpful as a routine diagnostic tool. For the time being, methylation-specific polymerase chain reaction appears to be the most useful method out of the ones available. Many cancers can be successfully treated if they are diagnosed early enough. Molecular markers can be used to further subclassify cancers by using the standard methods of diagnosis (cytology, histology, immunohistochemistry, serology, and so on). When it comes to tumour kinds and subtypes as well as chemotherapeutic response and survival, a tumor's methylation profile can help. Detection of cancer in its earliest stages can be improved by analysing methylation alterations. Furthermore, plasma can often be used to detect cancer cells in a sensitive manner. There are several types of tumours in the head and neck, as well as in the bladder, esophageal, gastric, esophageal, stomach, lung, prostate, and liver cancer. Additional samples that can be used include those collected using exfoliative cytology, endoscopic brush methods and biopsy as well as urine, saliva and sputum samples. In many cases, these methods are less invasive and thus less time-consuming to gather and analyse data. When using DNA methylation markers in cancer detection, sensitivity and specificity can be affected by a variety of factors, including the type of cancer and gene being researched, the type of bodily fluid used, and the technology used. Prior to clinical application, the assay must be standardised and demonstrated to be effective in a prospective manner (Kulis and Esteller, 2010).

Table 1: Detection of Cancer in Body Fluids Using DNA Methylation as Marker (Kulis and Esteller, 2010)

Tumor	**Gene**	**Specimen**	**Methylation**
Prostate cancer	*GSTP1*	Urine	36
		Serum	72
Non–small-cell lung cancer	*CDKN2A*	Sputum/Bronchial washings/BAL Serum	50 47

Breast cancer	Cyclin D2, *RAR*β,	Ductal lavage	85
	Twist gene *p16ink4a*	Serum	23
	p16, DAPK, MGMT, GSTP1	Serum	73
Head and neck cancer	*p16, DAPK, GSTP1* and *MGMT*	Serum	42

Saliva Hypermethylation as Diagnostic Biomarker in Oral Cancer

Promoter hypermethylation was initially discovered in 2007 when salivary DNA from patients with mouth cancer was examined. The promoter hypermethylation of APC, MGMT, ECAD p15, and p16 in oral malignancies, oral dysplasia, and normal controls was studied. 35 percent of oral cancer/dysplasia patients had methylation of p16, while MGMT and p15 had 29 percent methylation, APC had 14 percent methylation, and ECAD had 7 percent methylation. MGMT and p15 were found to have a 62.5 percent correlation with p16, whereas p16 and ECAD were shown to have an 87.5 percent correlation (Rapado-González et al., 2021).

Over the course of the study, 301 putative tumour suppressor genes were revealed to be significantly hypermethylated in oral SCC, while 92 genes were found to be hypermethylated in leukoplakia and 143 genes were found to be hypermethylated in tumour and leukoplakia tissues. These eight genes (EDNRB, HOXA9, GATA4, NID2 MCAM KIF1A DCC CALCA) were selected for validation in 24 oral cancer and 12 normal tissue samples from the discovery cohort for quantitative methylation-specific PCR (qMSP). Genetic methylation variations between patients and controls were found for EDNRB, HOXa9, GATA4, NID2, KIF1A, and DCC genes It was found that HOXA9 and NID2 were highly accurate in detecting head and neck cancer (85% sensitivity and 97% specificity) in an independent cohort of 55 tumours and 37 normal tissues. Patients with oral cancer, those with oropharyngeal cancer, and healthy controls were all analysed for the methylation status of the HOXA9 and NID2 genes in their salivary rinses. An AUC of 0.75 was found for HOXA9, and an AUC of 0.73 was found for NID2 when ROC curve analysis was performed to discriminate oral cancer patients from healthy controls. The combination of two salivary genes (HOXA9 + NID2) boosted the ability to distinguish between different types of food (AUC of 0.77). Both genes were found to be less sensitive when it comes to salivary cancer detection, which may be due to the different pathogenesis of oropharyngeal cancer, in which the human papillomavirus infection is the key

risk factor. As a result, the amount of tumoral cells in saliva may be lower than that in the oropharyngeal cavity, according to the authors. The Infinium HumanMethylation450 BeadArray was utilised by Langevin et al. to identify and verify a methylation classifier based on 22 CpG islands from oral rinses from 154 oral and pharyngeal cancer patients and 72 healthy controls. This saliva-based methylation biomarker panel is highly accurate in detecting oral and pharyngeal carcinomas, as indicated by its AUC of 0.92 (Langevin et al., 2015). For example, CDKN2A, RASSF1A, MGMT, DAPK1, and ECAD have all been examined in saliva DNA-methylation investigations for the presence of promoter hypermethylation. Eight genes (ECAD, MGMT, DAPK, RAR, p16, TMEFF2, WIF-1, and FHIT) had considerably higher levels of methylation in tumours compared to normal salivary samples, as reported by Nagata et al. As a result of this study, the salivary 4-gene panel had 100% sensitivity and 87.5 percent specificity, whereas 3-gene salivary panels had sensitivity and specificity values that ranged from 90% to 97%, respectively. Salivary promoter DNA methylation could help detect oral cancer without the need for invasive procedures (Nagata et al., 2012).Cancer suppressor gene p16/CDKN2A is frequently altered during oral cancer development (Padhi et al., 2017). The aberrant CDKN2A promoter hypermethylation identified in head and neck cancer research has been connected to tumour progression (Rapado-González et al., 2021).

There were significant epigenetic modifications linked with CDKN2A inactivation found in salivary mouth rinses from 10 oral cancer patients and three healthy controls, according to Kusumoto et al. CDKN2A expression was low or non-existent in four patients despite the presence of DNA promoter methylation and/or histone modification. According to these findings, CDK2A expression is influenced by a number of epigenetic modifications (Kusumoto et al., 2012). Ferlazzo et al. examined p16 and MGMT promoter methylation in saliva from 58 patients with oral cancer and 90 healthy controls in another study. There was a significant increase in the methylation of either P16 or MGMT promoters in oral cancer patients as compared to healthy controls (44.8 percent vs. 13.4 percent). Comparing individuals with MTHFR CT/AC or TT/AA genotypes to those with normal MTHFR genotypes, those with p16 promoter methylation were more likely. However, there was no statistically significant variation in the frequency of MGMT methylation. Our findings suggest that MTHFR mutations may play an important role in oral cancer, but more study is needed to determine whether or not they affect the gene-specific methylation process (Ferlazzo et al., 2017). MGMT promoter hypermethylation, which is connected to DNA repair, is common in individuals with head and neck cancer (Dvojakovska et al., 2018).

Utilizing methylation-specific PCR (MSP) and densitometry analysis, Liyanage et al. evaluated promoter hypermethylation using a panel of four tumour suppressor genes in saliva from 54 oral and 34 oropharynx cancer patients and 60 healthy controls. Promoter hypermethylation was seen in saliva from patients with cancers of the mouth and oropharynx, but no substantial methylation was found for p16. p16 and RASSF1A gene promoter hypermethylation was found to be related with advanced and high-grade oral cancer tumours, respectively. In addition, p16, RASSF1A, and TIMP3 hypermethylation was found to be associated with high-grade oropharynx cancers. Although p16 and RASSF1A were related with alcohol and tobacco intake, the promoter hypermethylation of the four tumour suppressor genes was connected with betel quid chewing. External variables such as smoke and alcohol may influence oral cancer development by altering DNA methylation processes. It also showed 91.7 percent sensitivity and 92.3 percent specificity for oral and oropharynx cancer, respectively, when paired with the four methylation genes in the study. These findings show that the salivary promoter hypermethylation gene panel has excellent discriminating value for both cancers (Liyanage et al., 2019).

It is known that TIMP3 is an extracellular matrix-bound protein that controls the activity of matrix metalloproteinases and inhibits the proliferation of cancer cells as well as their ability to migrate, invade, and create new angiogenesis. TIMP3 promoter methylation has been linked to the spread of oral cancer (Su et al., 2019). For the regulation of protein-coding gene expression connected to transforming growth factor signalling in head and neck cancer, the PCQAP/MED15 is a transcriptional coactivator mediator (Shaikhibrahim et al., 2015). There are numerous apoptotic and cell cycle checkpoint mechanisms regulated by the RASSF1A tumour suppressor gene (Dammann et al., 2017).

Among patients with oral cancer, promoter methylation of two tumour suppressor genes (p16 and RASSF1A) is much more common (44.2 percent and 23.3 percent, respectively) than in healthy controls (10 percent and 2 percent, respectively). When the two genes were combined, a significant level of specificity was detected, however the sensitivity of methylation detection was only 53.5 percent. Patients with advanced clinical stages, poorly differentiated tumours, and severe cellular abnormalities were found to have p16 and RASSF1A-methylation (González-Pérez et al., 2020). Saliva Hypermethylation as Prognostic Biomarker in Oral Cancer Numerous studies have demonstrated the prognostic value of gene promoter hypermethylation in several types of cancer. However, few studies have evaluated DNA

methylation markers in saliva for predicting prognosis in head and neck cancer (Bjerre et al., 2019).

For the detection of oral cancer, DNA methylation biomarkers have been reported to be sensitive and specific in their detection. Methylation microarray data has been used in recent studies to uncover new methylation markers for oral cancer diagnostic purposes. In the Gene Expression Omnibus library, Puttipa nyalears et al. identified 27,578 CpG sites and discovered that the TRH gene showed the largest methylation level differential between healthy cells and head and neck malignant cells. Pyrosequencing confirmed the presence of TRH site-specific methylation in healthy and malignant cells, with healthy cells showing 7 percent to 3.43 percent and diseased cells showing 63% to 19.81% of methylation. The TRH methylation in salivary oral rinses and oral swabs from a discovery cohort of 23 oral cancer patients and 33 healthy controls was also high, as demonstrated by quantitative real-time PCR. TRH methylation (AUC = 0.93) was found to be highly discriminating in oral rinses from 42 oral cancer patients and 54 healthy controls. Additionally, oral rinses containing methylated TRH (AUC = 0.88) can distinguish oropharyngeal cancer patients from healthy controls with 82.61% specificity (AUC = 0.88). In addition, this gene was found to have an 86.15 percent sensitivity, an 89.66 percent specificity, and an AUC of 0.93, indicating that this biomarker might be used to identify oral cancer (Puttipanyalears et al., 2018). CpG cg22881914 of the NID2 gene was selected for further examination by the same research team. It was determined that 43 oral cancer patients, 40 tobacco smokers, and 50 healthy controls have NID2 methylation in salivary oral rinses. NID2 salivary methylation was found to be significantly higher in oral cancer patients than in smokers and healthy individuals. NID2 methylation in oral swabs from matched oral cancer patients (n = 22) boosted sensitivity to 90.91 percent, which may be due to the greater quantity of epithelial cells from cancer lesions in oral swabs compared to salivary oral rinses (Srisuttee et al., 2020).

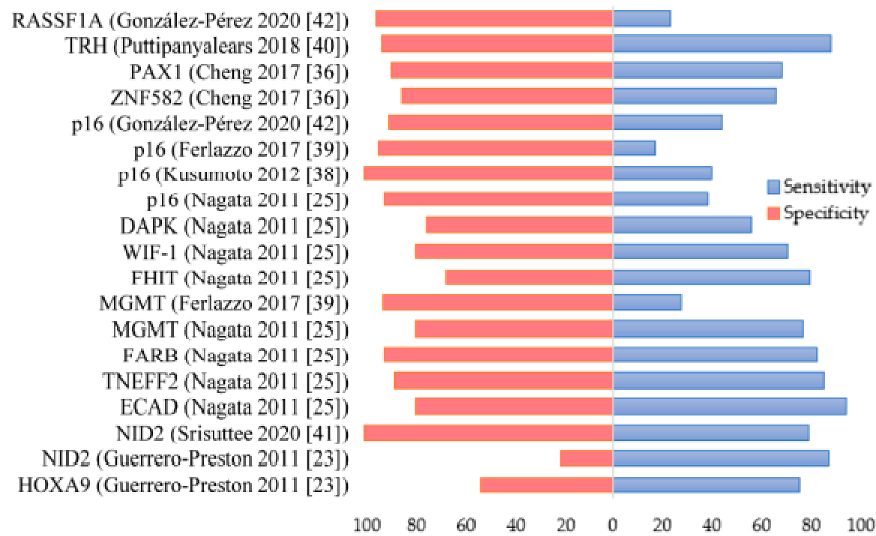

Figure 2: Reported sensitivities and specificities of DNA methylation biomarkers for the detection (Srisuttee et al., 2020).

Conclusion

More extensive research is needed to confirm the potential of salivary DNA methylation as a biomarker for oral cancer. Despite the evidence that salivary DNA methylation may be useful in the treatment of oral cancer, further research is needed before these epigenetic assays may be used in clinical oncology.

Reference

Asokan, G., Jeelani, S., and Gnanasundaram, N. (2014). Promoter hypermethylation profile of tumour suppressor genes in oral leukoplakia and oral squamous cell carcinoma. *Journal of clinical and diagnostic research: JCDR* 8**,** ZC09.

Baylin, S.B., and Jones, P.A. (2011). A decade of exploring the cancer epigenome—biological and translational implications. *Nature Reviews Cancer* 11**,** 726-734.

Bjerre, M.T., Strand, S.H., Nørgaard, M., Kristensen, H., Rasmussen, A.K., Mortensen, M.M., Fredsøe, J., Mouritzen, P., Ulhøi, B., and Ørntoft, T.J.I.J.O.M.S. (2019). Aberrant DOCK2, GRASP, HIF3A and PKFP hypermethylation has potential as a prognostic biomarker for prostate cancer. 20**,** 1173.

Dammann, R.H., Richter, A.M., Jiménez, A.P., Woods, M., Küster, M., and Witharana, C.J.I.J.O.M.S. (2017). Impact of natural compounds on DNA methylation levels of the tumor suppressor gene RASSF1A in cancer. 18**,** 2160.

Dvojakovska, S., Popovic-Monevska, D., Grcev, A., Pancevski, G., Benedetti, A., Popovski, V., Dimovski, A., and Stamatoski, A.J.J.O.C.-M.S. (2018). Promotor hypermethylated genes: Prospective diagnostic biomarkers in oral cancerogenesis. 46**,** 1737-1740.

Ferlazzo, N., Currò, M., Zinellu, A., Caccamo, D., Isola, G., Ventura, V., Carru, C., Matarese, G., and Ientile, R. (2017). Influence of MTHFR genetic background on p16 and MGMT methylation in oral squamous cell cancer. *International journal of molecular sciences* 18**,** 724.

González-Pérez, L.-V., Isaza-Guzmán, D.-M., Arango-Pérez, E.-A., Tobón-Arroyave, S.-I.J.J.O.C., and Dentistry, E. (2020). Analysis of salivary detection of P16INK4A and RASSF1A promoter gene methylation and its association with oral squamous cell carcinoma in a Colombian population. 12**,** e452.

Hentze, J.L., Høgdall, C.K., and Høgdall, E.V. (2019). Methylation and ovarian cancer: Can DNA methylation be of diagnostic use? *Molecular and clinical oncology* 10**,** 323-330.

Jin, B., Li, Y., and Robertson, K.D. (2011). DNA methylation: superior or subordinate in the epigenetic hierarchy? *Genes and cancer* 2**,** 607-617.

Kim, S.Y., Han, Y.K., Song, J.M., Lee, C.H., Kang, K., Yi, J.M., and Park, H.R. (2019). Aberrantly hypermethylated tumor suppressor genes were identified in oral squamous cell carcinoma (OSCC). *Clinical epigenetics* 11**,** 1-12.

Kulis, M., and Esteller, M. (2010). DNA methylation and cancer. *Advances in genetics* 70**,** 27-56.

Kusumoto, T., Hamada, T., Yamada, N., Nagata, S., Kanmura, Y., Houjou, I., Kamikawa, Y., Yonezawa, S., and Sugihara, K. (2012). Comprehensive epigenetic analysis using oral rinse samples: a pilot study. *Journal of oral and maxillofacial surgery* 70**,** 1486-1494.

Langevin, S.M., Eliot, M., Butler, R.A., Cheong, A., Zhang, X., Mcclean, M.D., Koestler, D.C., and Kelsey, K.T. (2015). CpG island methylation profile in non-invasive oral rinse samples is predictive of oral and pharyngeal carcinoma. *Clinical epigenetics* 7**,** 1-12.

Liyanage, C., Wathupola, A., Muraleetharan, S., Perera, K., Punyadeera, C., and Udagama, P.J.B. (2019). Promoter hypermethylation of tumor-suppressor genes p16INK4a, RASSF1A, TIMP3, and PCQAP/MED15 in salivary DNA as a quadruple biomarker panel for early detection of oral and oropharyngeal cancers. 9**,** 148.

Marur, S., and Forastiere, A.A. (Year). "Head and neck squamous cell carcinoma: update on epidemiology, diagnosis, and treatment", in: *Mayo Clinic Proceedings*: Elsevier), 386-396.

Nagata, S., Hamada, T., Yamada, N., Yokoyama, S., Kitamoto, S., Kanmura, Y., Nomura, M., Kamikawa, Y., Yonezawa, S., and Sugihara, K. (2012). Aberrant DNA methylation of tumor-related genes in oral rinse: A noninvasive method for detection of oral squamous cell carcinoma. *Cancer* 118**,** 4298-4308.

Ovchinnikov, D.A., Cooper, M.A., Pandit, P., Coman, W.B., Cooper-White, J.J., Keith, P., Wolvetang, E.J., Slowey, P.D., and Punyadeera, C. (2012). Tumor-suppressor gene promoter hypermethylation in saliva of head and neck cancer patients. *Translational oncology* 5**,** 321-326.

Padhi, S.S., Roy, S., Kar, M., Saha, A., Roy, S., Adhya, A., Baisakh, M., and Banerjee, B. (2017). Role of CDKN2A/p16 expression in the prognostication of oral squamous cell carcinoma. *Oral oncology* 73**,** 27-35.

Powrozek, T., and Malecka-Massalska, T. (2016). DNA hypermethylation of tumor suppressor genes as an early lung cancer biomarker. *Translational cancer research* 5**,** S1531-S1538.

Puttipanyalears, C., Arayataweegool, A., Chalertpet, K., Rattanachayoto, P., Mahattanasakul, P., Tangjaturonsasme, N., Kerekhanjanarong, V., Mutirangura, A., and Kitkumthorn, N.J.B.C. (2018). TRH site-specific methylation in oral and oropharyngeal squamous cell carcinoma. 18**,** 1-9.

Rapado-González, Ó., López-Cedrún, J.L., López-López, R., Rodríguez-Ces, A.M., and Suárez-Cunqueiro, M.M. (2021). Saliva Gene Promoter Hypermethylation as a Biomarker in Oral Cancer. *Journal of Clinical Medicine* 10**,** 1931.

Shaikhibrahim, Z., Offermann, A., Halbach, R., Vogel, W., Braun, M., Kristiansen, G., Bootz, F., Wenzel, J., Mikut, R., and Lengerke, C.J.T.a.J.O.P. (2015). Clinical and molecular implications of MED15 in head and neck squamous cell carcinoma. 185**,** 1114-1122.

Shi, H., Chen, X., Lu, C., Gu, C., Jiang, H., Meng, R., Niu, X., Huang, Y., and Lu, M. (2015). Association between P16INK4a promoter methylation and HNSCC: a meta-analysis of 21 published studies. *PloS one* 10**,** e0122302.

Srisuttee, R., Arayataweegool, A., Mahattanasakul, P., Tangjaturonrasme, N., Kerekhanjanarong, V., Keelawat, S., Mutirangura, A., and Kitkumthorn, N.J.B.C. (2020). Evaluation of NID2 promoter methylation for screening of Oral squamous cell carcinoma. 20**,** 1-9.

Struhl, K. (2014). Cancer: Is DNA methylation of tumour suppressor genes epigenetic? *Elife* 3**,** e02475.

Su, C.-W., Chang, Y.-C., Chien, M.-H., Hsieh, Y.-H., Chen, M.-K., Lin, C.-W., Yang, S.-F.J.C.D., and Disease (2019). Loss of TIMP3 by promoter methylation of Sp1 binding site promotes oral cancer metastasis. 10**,** 1-17.